Indian J. Phys. **77B** (1), 113-123 (2003)

AN INTERNATIONAL JOURNAL
IJP B

Raman spectroscopy in clinical investigations

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Abstract Raman spectroscopy has been successfully applied in several areas of biology and medicine, including diagnosis of malignancy The applications of Surface Enhanced Raman spectroscopy and micro-Raman have improved to the extend of studying single molecule dynamics and cellular biochemistry respectively Raman spectroscopy studies carried out in our laboratory on oral cancer, osteoradionecrosis, radiation induced damages in mouse models are presented and discussed We have recorded Raman spectra of normal and malignant oral tissues and the obtained spectra were analysed using statistical (PCA) methods An objective diagnosis method with high sensitivity and specificity based on Mahalanobis distance and spectral residual is developed for oral malignancy The study of radiation induced damage in mouse brain and muscle tissue suggests that radiation activated chemical cascade is similar to those in stress, but it persists for longer periods Radiation treatment on bone leads to immediate structural changes in the mineral part of the bone

Keywords Raman spectroscopy, SERS, oral cancer, PCA analysis, radiation induced damage, ORN bone

PACS Nos 78 30 Am, 87 50 Hj, 87 64 Je

1. Introduction

The discovery of Raman effect in the year 1928 demonstrated that the analysis of inelastically scattered light from the simplest molecule H_2O, can provide unique finger print of molecular structure [1, 2]. In the last 75 years, popularity and versatility of Raman scattering spectroscopy have increased in many ways and a diverse family of Raman-based techniques has been developed. More and more sensitive experimental approaches continue to be developed to explore the molecular mechanisms of complex biological phenomena. Raman spectroscopy has also been identified as a reliable diagnostic technique [3-5]. A larger number of biological molecules can be probed by using Raman spectroscopy. Several studies show the potential of near-infrared Raman spectroscopy for the detection of cancer and pre-cancer *in vitro / in vivo*, as a new tool [3-5].

UV Resonance Raman scattering selectively increases the scattering signal from the ground state vibration modes that are coupled to excited vibronic levels [6]. This large enhancement in the Raman scattering cross section of specific molecular vibration modes, offers great advantages over non-resonance Raman scattering. Research findings show that UVRR spectroscopy can be used to characterize normal and diseased colon tissue by selectively enhancing spectra of aromatic amino acids, and parameterizing their contribution to the colon spectrum That means, UV RR spectroscopy can provide complete biochemical characterization of the tissue under study as well as it can describe the pathological change [6].

Micro-Raman spectroscopy is a powerful tool for study of the structural variations in samples of sizes down to sub microns [7, 8]. In the Raman microanalysis, a laser beam is focused onto a very small area with a microscope objective and Raman scattered light from the area is collected by the same objective, dispersed by a monochromator and spectra recorded. Raman microscopy has potential utility in structural studies *in situ*. Recent advances in lasers, detectors, and spectrograph and filter technologies have made it possible to detect even very weak Raman signals from a single living cell [7, 8].

Ultrasensitive Raman detection based on surface enhanced Raman scattering is now well established [9, 10]. Surface-enhanced Raman spectroscopy (SERS) is a phenomenon resulting in strongly increased Raman signals of molecules that

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have been attached to nanometer sized metallic structures. Several papers have been appeared discussing and demonstrating the mechanism of SERS [9-15]. It has been used to study the single-molecules Raman spectra of biomolecules with smaller sizes [10-14] Katrin Kneipp and collaborators demonstrated that NIR SERS on colloidal silver clusters is an excellent technique for single molecule detection that is applicable for a broad range of molecule including colourless biomolecules These group presented the detection of adenine molecule without any labeling based on its intrinsic surface-enhanced Raman scattering [15] SERS has also been extended towards understanding of intermolecular interactions [16]

In living systems, the cells are held together by extra cellular macromolecules like collagen Depending on the location of the tissue, there are various kinds of tissues like epithelial, connective, smooth muscle *etc*. That means, the composition of tissue and cells may vary from one type to another Further, the cytoplasm of the cell contains various molecular systems like lipids, DNA, enzymes, NADH, heme particles *etc* and these constituents are distributed inhomogeneously. Therefore, Raman spectroscopy of biological tissues is quite different from other samples. Due to the fluorescence emission cross section for a typical fluorophore being of the order of a million times larger than that of Raman scattering, fluorescence usually mask the weak Raman signals However, this problem nowadays can be eliminated by exciting the tissue with wavelengths longer than 700 nm (very low fluorescence) or UV wavelengths lower than 300 nm [19], when there is no fluorescence interference in the Raman region

In our laboratory we have been using normal, micro and SERS Raman spectroscopy for biomedical applications We have carried out Raman spectral studies on soft tissue, bone, drugs and body fluids. Some of our results are presented and discussed in this paper.

2. Materials and methods

Raman spectroscopy of biological samples studied here covers biomolecules, tissues, body fluids and bone. The tissue, bone and other physiological samples were obtained from various departments of Kasturba Medical College, Manipal. As explained earlier, the Raman cross section can be increased enormously by using the technique of surface enhanced Raman scattering. For our SERS experiments, we have used silver colloid as the metal support to achieve signal enhancement. Various chemicals like dopamine, $AgNO_3$, $NaBH_4$, and Polyvinyl alcohol were purchased from Merck (India) and used as received. Colloids for SERS were prepared with minor modifications of the method of Lee and Meisel [20]. The samples were excited with 785 nm diode laser and the resulting surface enhanced Raman spectra recorded.

3. Experimental setup

The experimental Raman setup (Figure 1) consists of a single-stage imaging spectrograph (ISA Jobin-Yvon Spex HR-320, f/4.1) fitted with a liquid nitrogen cooled CCD detector (Spectrum One, 2000 × 800 pixels with an active area of 30 × 12 mm). The spectra were recorded with a 600 g/mm grating blazed at 1 μm, with a slit width of 100 μm (7 cm $^{-1}$). NIR laser excitation of 785 nm was provided by an SDL-8530 diode laser. Af f1 lens was used for collection of scattered light. The scattered laser light was removed using an HSPF-5812 super notch filter (Kaiser Optical Systems Inc) in the collimated beam, and Raman scattered radiation was focussed onto the spectrograph slit with an f/4 lens.

4. Results and discussion

(a) Raman spectroscopy of oral cancer tissues :

Oral cancer is one of the 10 most common cancers in the world with more than 500000 cases projected world wide annually [21]

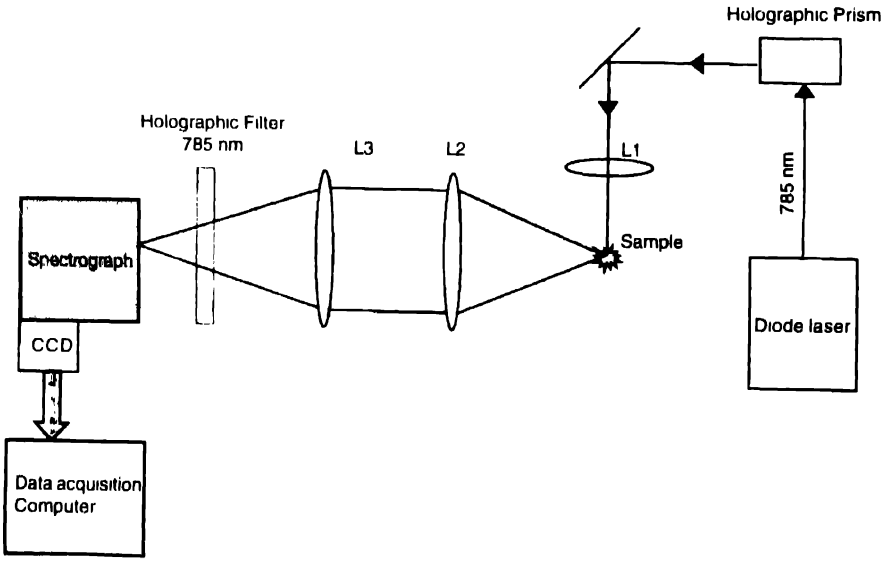

Figure 1. Schematic diagram of Raman spectroscopy system

Incidence of this form of cancer is very high – accounting for as many as 50% of all cancers [22] – in India and other Asian countries which is attributed to chewing of tobacco and reverse smoking. Malignant tumors of the lip, oral cavities, and pharynx are the most commonly cited group of cancers in the Indian cancer registry [23]. Survival after treatment (surgery and/or Radiation therapy) over 5 years is as low as 55%, which is attributed to late detection and short comings of existing method of detection- histopathological examination [24, 25]. Optical spectroscopy based pathology methods have been suggested as potential alternative [26]. Applications of Raman spectroscopy in histochemical analysis for breast, cervical and other forms of malignancies have been reported and Reference 27 provides a good review of these methods. To the best of our knowledge we are the first to do detailed Raman spectroscopy studies in oral cancer.

Raman spectra of normal and malignant tissue specimens are strikingly different as shown in Figure 2. In general the spectra of normal tissue samples resemble lipids, (C = C, C = O, C–C), while the malignant tissue samples show a more protein like spectra (Amide I, Amide II, Phenylalanine). Curve fitting of the spectra in 1000–1750 cm $^{-1}$ region was done in order to understand the biochemical changes and the results are shown in Figure 3. Assignment of spectra of the normal and malignant tissues is presented in Table 1, which gives us clear evidence of

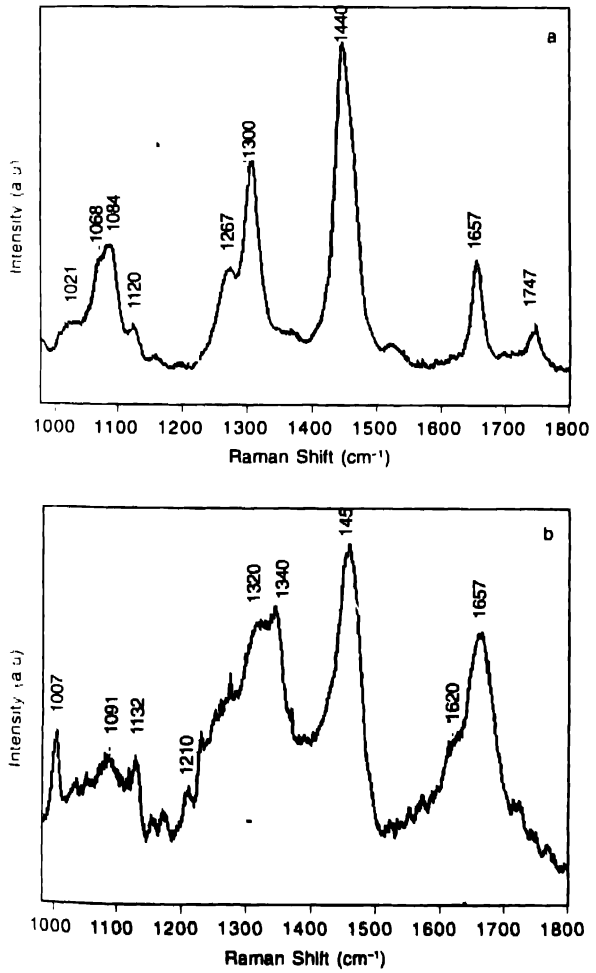

Figure 2. Raman spectra of buccal mucosa. (a) Normal and (b) Malignant.

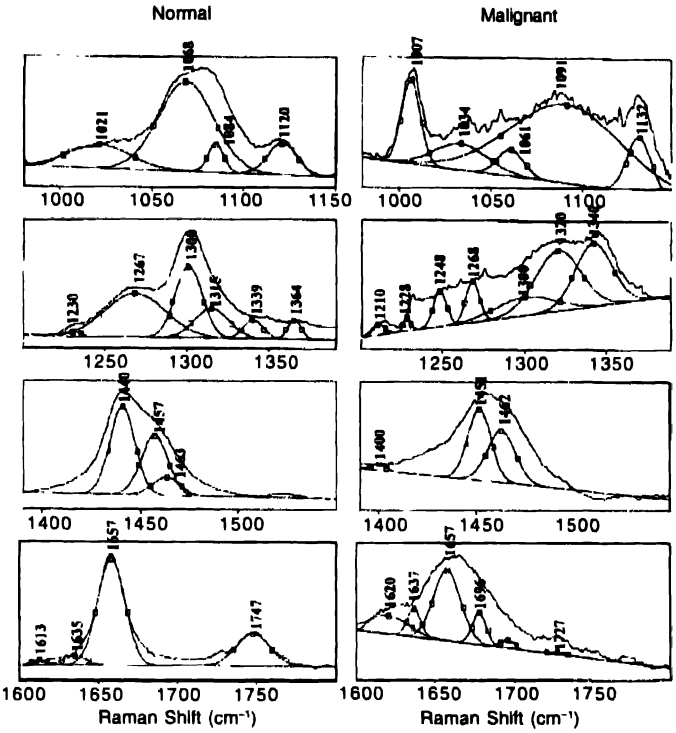

Figure 3. Curve fitted Raman spectra of normal and malignant oral tissue

biochemical alterations in cancer tissue. Figure 4 shows the Raman spectra across a sample which had been diagnosed pathologically, as malignant at the center, the edges being normal. The spectral characteristics show the corresponding change from malignant to normal, as we scan across the sample. Thus this technique has many medical and surgical applications,

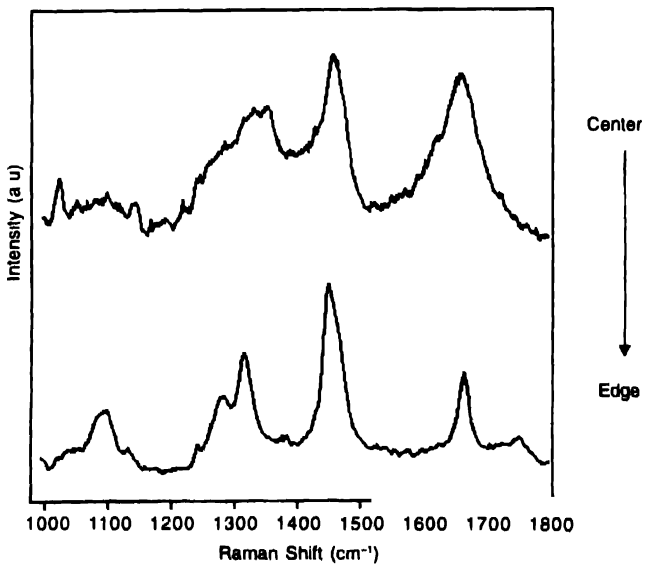

Figure 4. Raman spectra recorded across a surgically resected sample.

Table 1. Frequencies and assignments of major vibrational bands in the Raman spectra of oral tissues after curve fitting

Raman Shift		Assignments
Normal	Malignant	
	1007 strong, sharp	ν (C-C) Phe, ring breathe
1021 weak	–	ν_a (C-C-N$^+$)
	1034 weak, broad	Phe
	1061 weak	ν (C-N)
1068 strong, broad		ν $(C\text{-}C)_{trans}$ phospholipid
1084 weak		ν $(C\text{-}C)_{gauche}$
	1091 medium broad	ν (C-N)
1120 weak		ν $(C\text{-}C)_{trans}$
	1132 medium	ν (C-N)
	1210 very weak	Try and Phe modes
1240 very weak	1228 very weak	PO$_2^-$ ant Symmetric Stretch
	1248 weak	Amide III (β-chain + random coil)
1267 medium	1268 weak	δ (= CH) Phospholipid
1300 strong, sharp	1300 weak	τ $(CH_2)_n$ lipids
1315 medium	1320 strong	Amide III (α-helix)
1339 weak	1340 strong	δ (CH) residual vibrations
1364 weak	–	CH_3 Symmetric Deformation
	1400 very weak	ν (COO$^-$)
1440 very strong		δ $(CH_2)_n$ lipids
1457 weak	1451 very strong	δ_{as} (CH_3) and δ (CH_2) of proteins and lipids
	1462 medium, broad	δ (CH_2) (CH_3)
1463 weak		CH_2sci
1613 very weak	1620 weak	Trp (IgG), Tyr and Phe
1635 very weak		ν water
	1637 weak	Amide I (Both α-helix and β structure)
1657 strong, sharp		ν (C = C) cis, phospholipid
	1657 very strong, broad	Amide I (α - helix)
	1696 weak	Amide I (turns and bends)
	1724 very weak	ν (C = O) proteins
1747 medium, shar		ν (C = O) lipids

Trp Tryptophan, Tyr Tyrosine, Phe-Phenylalanine, δ-bending, ν-stretching, τ twist

like screening of general population, decision making in surgical procedures, follow up for regression or recurrence, and monitoring effectiveness of therapy. A biopsy followed by pathological examination is not so convenient in all the above cases, because of the need to remove and examine the tissue, which takes considerable time. Raman spectra can be successfully used as a routine probe. With a fiber optic probe, spectra can be obtained *in situ* in a few minutes and this facilitates all the applications mentioned above. To illustrate this, typical Raman spectra of normal oral tissue obtained using fiber optic probe is shown in Figure 5.

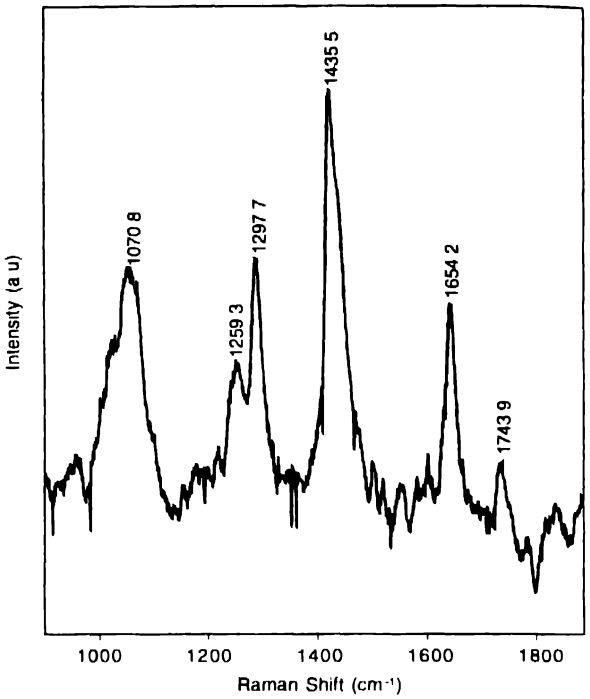

Figure 5. Raman spectra of normal oral tissue recorded using a fiber optic probe

One of the aims of optical based pathology is to develop methods which are highly objective and reduce requirement of highly qualified personnel like certified pathologists. To achieve an objective evaluation of spectra as a discrimination tool, we used statistical methods and Principal Component Analysis (PCA) [29] for data analysis. In PCA, a large number of spectra are expressed in terms of few factors which account for the variations. Contribution of individual factors in each spectrum are known as scores. Using factors and scores, entire spectra can be reconstructed. Scores of the factors have been widely used as discrimination parameters. But in many cases this method may not provide good discrimination. Figure 6 shows the result of combining all the data (normal and malignant) and using the score of factor 1 as a discrimination parameter. As can be seen from the figure it provides only reasonable discrimination. Alternately other parameters like spectral residuals (difference between simulated and actual spectra) can also be used for evaluation. We have employed the Mahalanobis distance (M-distance) [30] and the residual errors squared sum (Spectral Residual) as the criteria. The Mahalanobis distance for a sample is a function of all the scores for that sample, and when compared to a model set, will clearly show whether that sample belongs to the model set, with well defined statistical probability. Figure 7 shows a plot of the M-distance against Residual Errors Squared Sum for a new set of about 124 samples, compared to a model

set of 25 randomly selected malignant spectra. It is seen that samples diagnosed as malignant by pathological examination in the new set fall in the lower left hand corner of the plot. If we take an M-distance of 1 and residual 0.25×10^8 as acceptable,

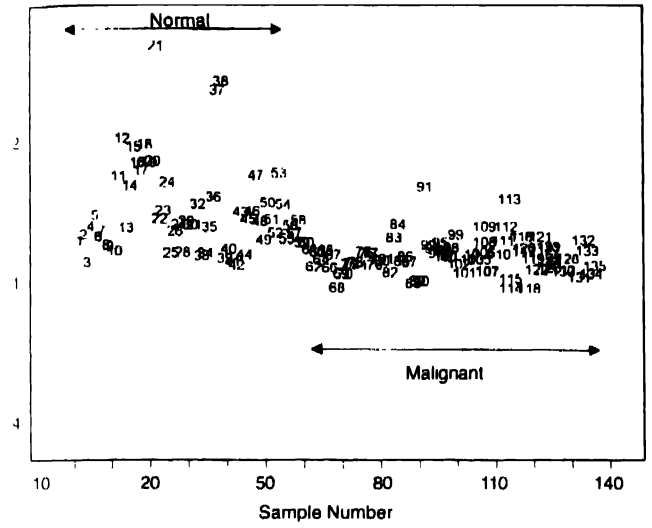

Figure 6. PCA of normal and malignant combined Plot of Score of Factor 1 vs Sample No

then almost all the malignant samples fall within 2 times this value, while most of the samples classified as normal lie outside. Based on Figures 6 and 7, combined use of M–distance and spectral residual seem to be better for discrimination. Moreover, by fixing acceptance value for M–Distance and residuals it is possible to verify test spectra for 'match or no match' with standard calibration set. This provides a highly objective diagnosis The sensitivity and specificity, based on 90 test spectra are better than 85 and 90 percent respectively [28]

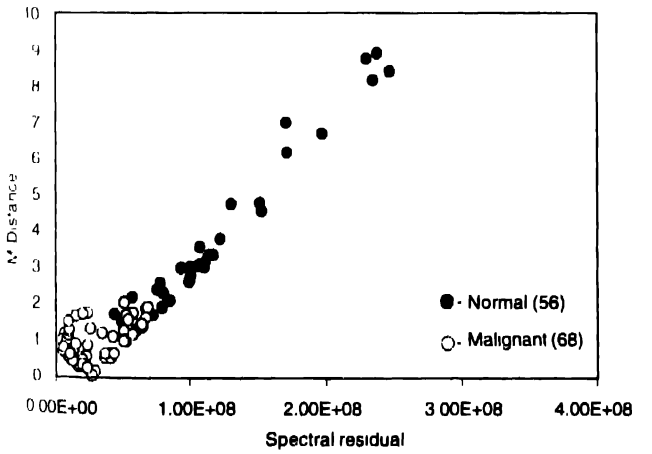

Figure 7. Classification of 124 spectra (62 malignant, 62 normal) compared to the model set of malignant spectra. A Mahalanobis Distance up to 3 can be considered as malignant

The observed spectral differences can be attributed to the changes in the surface molecules in cells of epithelial region. Though 785 nm, the excitation source, has very good penetration in the tissue, the collection of Raman scattering could be mostly from the surface region due to loss of scattered light from other layers. In our micro-Raman spectroscopy studies, we have selectively recorded spectra from epithelial and subepithelial regions. Significant spectral differences between normal and malignant tissue from epithelial region were noticed. On the other hand, no considerable differences could be noticed from subepithelial region [31], indicating that spectral differences between normal and malignant tissue originate from the epithelial region.

(b) SERS spectra of neurotransmitters :

SERS has emerged as one of the promising techniques for neurochemical studies [17]. Many brain disorders are directly linked with neurotransmitter release by the central nervous system [18]. SERS can quantify trace amounts of many biochemical molecules which in turn helps to get an insight in many physiological reactions. This detection and quantification of neurotransmitters, for example, can lead to an understanding of the brain activities and their role in brain disorders. We show in Figure 8 an SERS spectrum of dopamine, a neurotransmitter which is linked with Parkinson's disease.

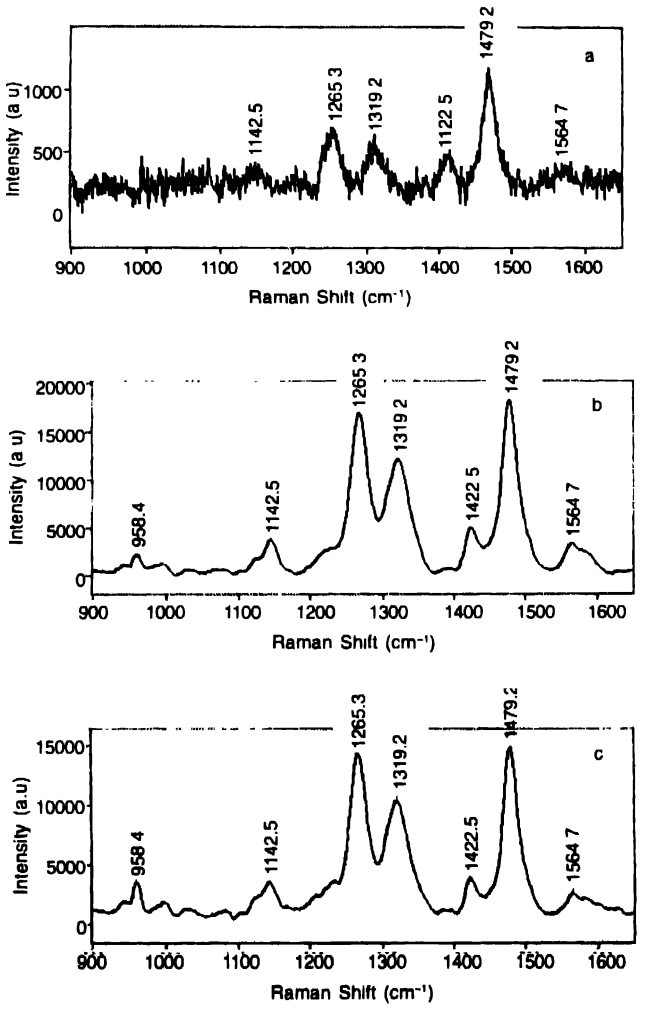

Figure 8. SERS spectra of dopamine (a) dopamine alone, (b) dopamine+ colloid and (c) dopamine+ colloid+ NaCl

It should be noted that the laser beam, about 3 mm in size, when focussed with 5 cm focal length lens gives a spot size of approximately 16-20μm. The sample was contained in a 1 mm diameter quartz capillary and the total volume illuminated is thus less than 300 pico litters giving the total amount of material less than 3 fm. Even $1/100^{th}$ of this amount can be easily detected as seen from the number of counts for the strong peak at 1479.2 cm^{-1}, giving a detection limit 30 atto moles.

Another example of the sensitivity of the technique is seen in spectra of "pure water" drawn through a freshly opened commercial plastic syringe, identical to those used in many medical applications) and a glass syringe, Figure 9. The SERS of water drawn from disposable syringe showed many peaks, probably from decomposition products of the materials used in manufacture of syringe, (Figure 9b) Same water, when drawn with a freshly cleaned Pyrex syringe did not show any SERS peaks (Figure 9a)

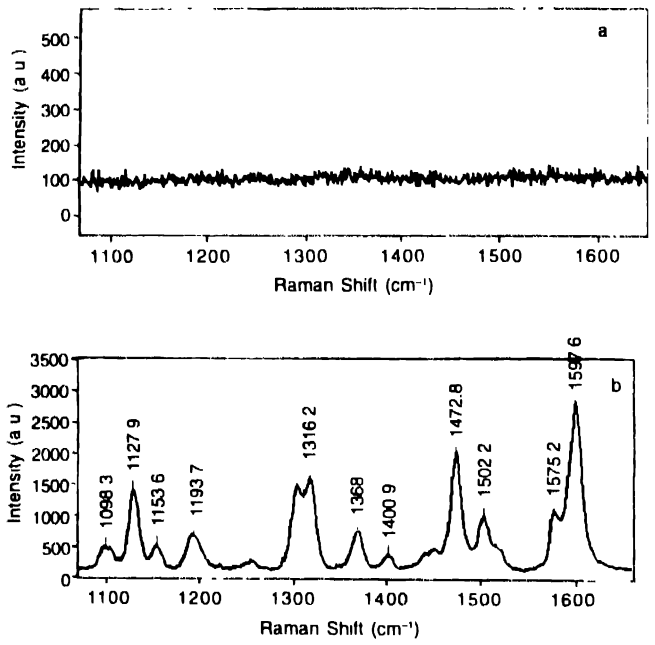

Figure 9. SERS spectra of (a) pure water drawn from plastic syringe + colloid + NaCl and (b) pure water drawn from pyrex syringe + colloid + NaCl

(c) Radiation-induced damages on brain irradiation in mouse models :

Radiotherapy is routinely employed as a major treatment modality in several forms of malignancy including cancers in central nervous system and head and neck cancers where the central nervous system is exposed to ionizing radiation. High mortality and morbidity is associated with the CNS injury that is induced due to radiotherapy. Because of this radioresponse of brain and spinal cord had been extensively investigated [32-35]. Based on time of expression, radiation induced CNS injury is divided into three categories, acute, early delayed and late delayed. Radiation damages of CNS, histologically, are not unique and quite similar to those of other types of injury. The pathways leading to these damage and repair can be described as acute cell death, radiation activated chemical cascades and induction of genes coding for protective factors like cytokines. It should be possible to follow these processes by monitoring biochemical interactions initiated by radiation injury. We have carried out Raman spectroscopy studies on mouse models subjected to brain irradiation to find out the biochemical changes brought about in tissue and brain, as a result of radiation injury. We have also included other forms of injuries like trauma and effect of anesthetic drugs. We have investigated both brain, the organ which received the radiation, and muscle tissue far removed from the irradiation site [36].

Random bred adult Swiss albino mice of 6-8 weeks, of either sex, weighing 25-30g were used in the experiments. Eight mice were divided into 2 sets of 4 mice each. Mice in each set were anaesthetized by injecting 50-mg/kg-body weight of Ketamine and 0.1ml of Calmpose into the peritoneal cavity. They were immobilized by putting them into perforated plastic tubes fixed uniformly in a perplex plate For one set, the bodies of mice were shielded with 4 half value layers of lead and their heads were exposed to 10 Gy γ radiation from Theratron – 780C (Cobalt teletherapy unit, Kasturba Hospital, Manipal Academy of Higher Education). The second set was sham irradiated. Animals were sacrificed at 2h, 24h, 48h and 1 week after the treatments In each set, at each time point only one animal was studied. The experiment was repeated at least 4 times (and in some cases even more) for each time point for both sets. To study the stress

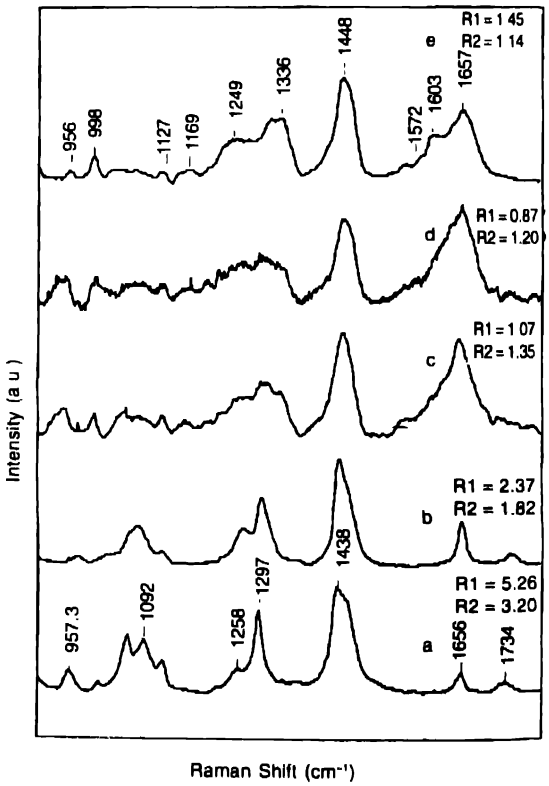

Figure 10. Raman spectra of (a) Palmitoyl oleoyl phosphatidyl choline, (b) Muscle tissue control, (c) Muscle tissue 30 min after stress, (d) Muscle tissue 30 min after exposure of brain to 10 Gy γ radiation and (e) Albumin $R_1 = I_{1440} / I_{1656}$, $R_2 = I_{1400} / I_{1250}$.

induced by retention and any trauma due to injections, mice were given 300 µl of doubly distilled water injection intraperitonially and immobilized in the restrainer for half an hour (same as the time of retention in group one). Further investigations were same as for the case irradiation. Control experiments were carried out on animals which were sacrifcied humanely by cervical dislocation. The studies were conducted according to our institutional regulations and national criteria for animal experimentation.

Samples of brain and muscle tissue kept moist with saline were subjected to Raman spectroscopy. As seen in Figure 10 brain irradiation and other forms of injuries like stress and administration of anaesthetics produces drastic spectral changes even in tissue locations far removed from the radiation site. The spectral changes are very similar to one another in all cases. However, the changes produced by stress or drug administration last only for a short time (few hours to one or two days), whereas radiation induced changes persist even after one week which can be seen in Figure 11. The spectral changes can be interpreted in terms of observation of new spectra, dominated by bands due to proteins. In contrast to muscle tissue, both white and gray matter of control brain tissue show a mixture of protein and lipid spectra, but the white matter shows a much larger amount of lipid spectra. This is clear from Figure 12, where the 1440/1660, and 1300/1250 ratios are larger for white matter (Figure 12a) compared to gray matter (Figure 12b). Figure 13 a and b show the spectra of gray and white matter half an hour after 10

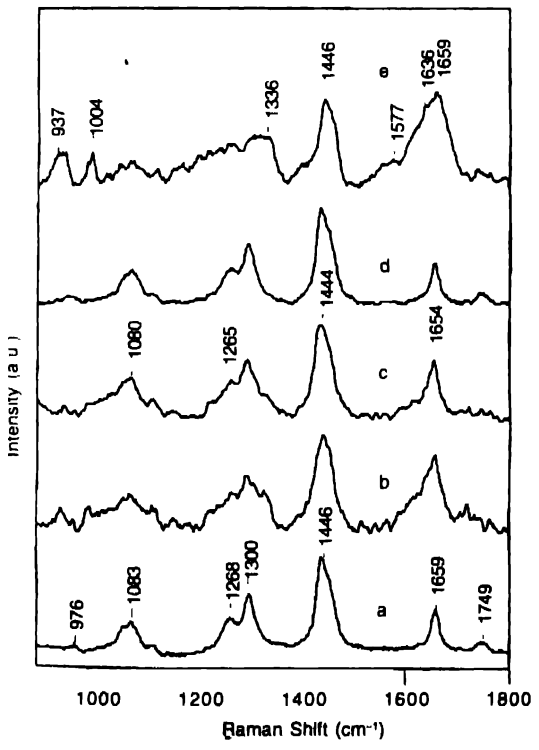

Figure 11. Raman spectra of muscle tissue. The spectra show the different recovery times after stress, administration of anesthetic and 10 Gy radiation to brain. (a) 2*h* after stress, (b) 24 *h* after administration of anaesthetic, (c) 48 *h* after administration of anaesthetic, (d) one week after administration of anesthetic and (e) one week after exposure of brain to 10 Gy γ radiation. $R_1 = I_{1440} / I_{1656}$, $R_2 = I_{1300} / I_{1250}$

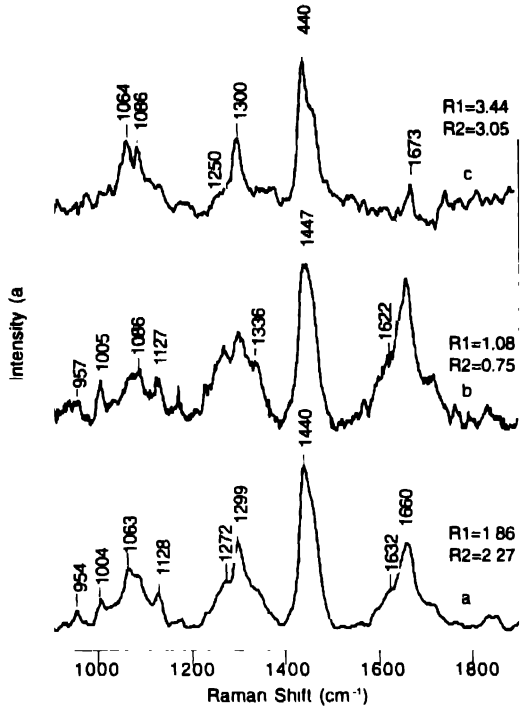

Figure 12. Raman spectra of control (a) Brain white matter, (b) Brain gray matter and (c) Difference spectrum (white–gray), after normalization of both spectra to 1660 cm^{-1} band The difference spectrum is similar to that of OPPC, Figure 10a

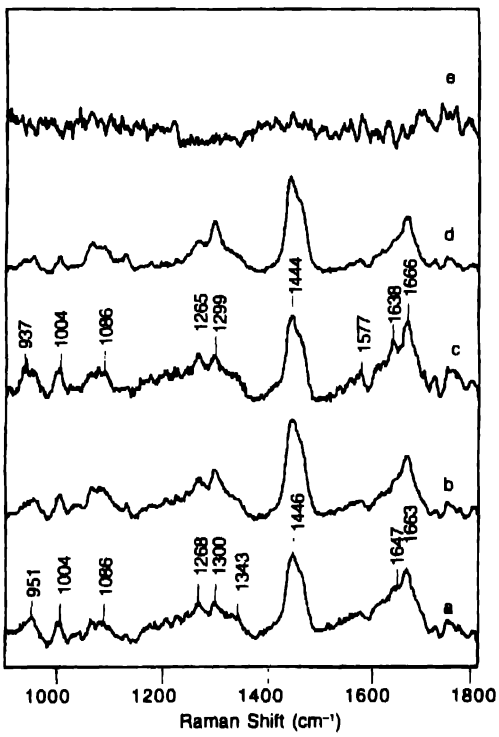

Figure 13. Raman spectra after exposure of brain to 10 Gy radiation (a) Gray matter after 30 min, (b) White matter after 30 min, (c) Gray matter after one week, (d) White matter after one week and (e) Difference spectrum (control Figure 12a–48 h after brain irradiation) for white mattter after normalization of both spectra with respect to 1660 cm^{-1}.

Gy irradiation. Comparing with Figure 13b and 12a respectively, it is seen that there is very little change in the spectra even immediately after irradiation. The main changes are a slight increase in intensities for bands in the 1500-1650 cm^{-1} region, the 1270 cm^{-1} band, and the 1004 cm^{-1} band, these changes being more prominent for gray matter As in the case of the muscle tissue, these differences persist even after one week (Figure 13c) for gray matter, while the white matter seems to recover fast (figure 13d). [36]

The results thus support the hypothesis that various protective factors are released through out the system on exposure of the Central Nervous System (CNS) to radiation. These protective factors like cytokines and enzymes continue to be produced in radiation damage even after several days, indicating that radiation activated chemical cascades, while similar to those in stress or administration of anesthetic, persist for much longer periods, contributing to early delayed and late delayed damages.

(d) Raman spectroscopy of bone .

Raman spectral studies on bone (bovine) has been carried out mainly from the point of view of analysis of mineral and collagen contents [37] and micro cracks developing as a result of strain [38]. As we mentioned in the study on radiation effects, radiation therapy for various forms of cancer, especially in the head and neck region, results in acute, early delayed, and late delayed effects One such effect is the onset of OsteoRadioNecrosis (ORN) in some patients who undergo radiation therapy for oral cancer. In ORN the mandibular bone decays, becomes brittle and get detached from the surrounding soft tissue causing severe damage The current hypothesis is that radiation therapy leads to hypoxic, hypovascular, hypocellular tissue where cell death and collagen lysis exceeds cell replacement and collagen synthesis. Bone tissue in adults is being continuously remodelled (dissolved and rebuilt), and this requires sustained activity of osteoblasts and osteoclasts. These cells are highly transient and are produced from the stem cells in bone marrow, together with adiposites [39]. We have studied the Raman spectra of normal bone, bone from subjects who have undergone radiation therapy but have not developed ORN, and bone which has developed ORN

Figure 14 shown that Raman spectra of the cortical and cancellar regions of normal bone, and bone after radiation therapy but without ORN The cortical region of bone contains about 70% dry weight of Calcium Hydroxy Apatite with traces of $CaCO_3$, the remaining 30% being collagen. The cancellar region contain bone marrow with all the associated cells like adiopocytes. It can be seen that the cortical region spectra, Figure 14a, shows strong mineral bands with weak bands of collagen, while the cancellar region contains strong lipid bands with mineral bands (Figure 14b).

The cortical and cancellar region of normal bone and bone after radiation therapy (no ORN) show small but significant changes. This important changes observed are a shift to higher frequency of the phosphate ν_1 band (955 cm^{-1} to 961 cm^{-1}), increase in intensities of the ν_2 (433 cm^{-1}) and ν_4 (590 cm^{-1}) bands and a slight decrease in the intensities of the lipid bands (1439 cm^{-1}, 1300 cm^{-1}, 1269 cm^{-1} and 1658 cm^{-1}). These results show that exposure to radiation causes an immediate change in the structure of the mineral part and causes some cell death The hydroxy apatite part of the bone bas become more crystalline as in samples which undergo strain [38]. The immediate cell death causes decrease in adipocytes and other cells, causing slight decrease in the intensity of the lipid bands.

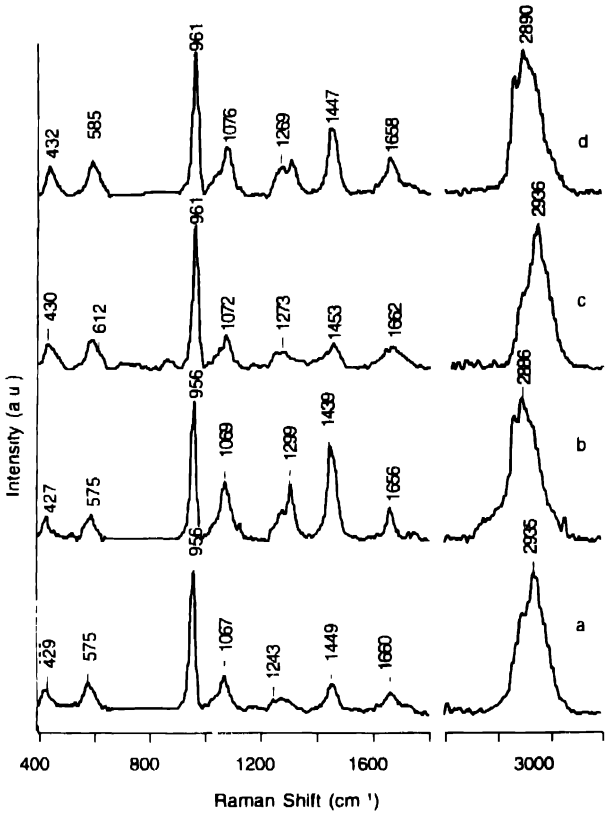

Figure 14. Raman spectra from (a) Normal bone cortical region, (b) Normal bone cancellar region, (c) Bone cortical region after radiation therapy and (d) Bone cancellar region after radiation therapy

Figure 15a shows the Raman spectrum of ORN bone. In ORN bone both cortical and cancellar regions gave the same type of spectra, and it is seen that practically all the lipid bands have disappeared. This is shown more clearly in Figure 15c, where the difference spectrum of cancellar and ORN bone is shown, *i.e.* Normal bone-ORN bone. All the lipid bands are left in the difference, showing absence of lipids in ORN. Similar decrease in lipid band intensities are also seen in the difference spectra (Normal bone cortical-ORN bone) as shown in Figure 15b. Figure 15b also indicates some loss of collagen in ORN as shown by the negative band at Amide 1 position.

The results show that radiation therapy leads to immediate structural changes in the mineral part of the bone and some cell death. If the cell death is controlled or cells are replenished, the

bone, though structurally changed, can rebuild itself, and return to normal condition. On the other hand if all the cells, including stem cells are lost, no remodeling takes place and permanent injury in the form of ORN is caused.

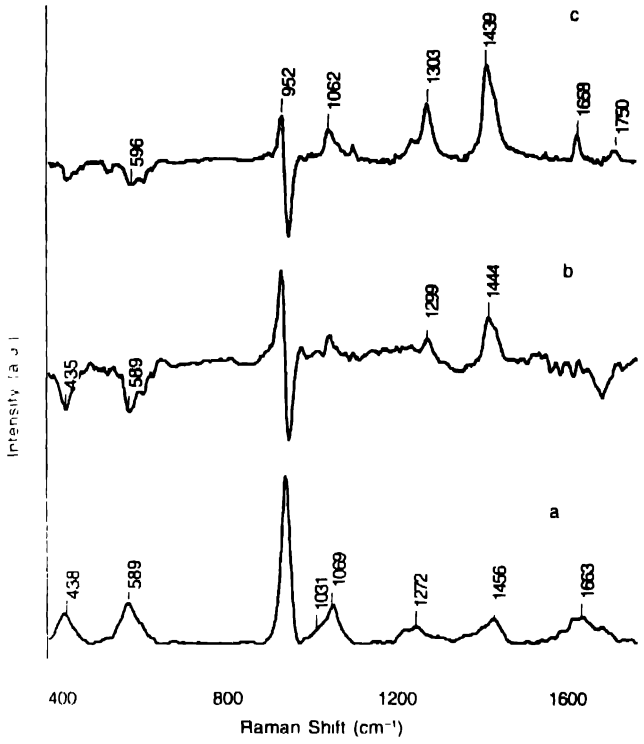

Figure 15. (a) Raman spectrum of ORN bone Raman difference spectra, (b) Normal bone cortical – ORN bone and (c) Normal bone cancellous – ORN bone

5. Conclusions

NIR Raman spectroscopy technique is very effective for various clinical diagnostic applications. It can be routinely used for optical pathology of tissue to diagnose malignancy. SERS spectroscopy can be used as an efficient tool for the detection of ultra trace quantities of molecules of biochemical significance. Raman spectroscopy studies on irradiated rat brain have shown that acute injury of the CNS by radiation results in various cellular and biochemical reactions that activate signaling processes leading to early delayed and late delayed changes. Raman spectroscopy has shown that such early delayed and late delayed changes may underlie some of the serious after effects of Radiation Therapy, like ORN.

Acknowledgment

The authors are grateful to Department of Science and Technology and Department of Atomic Energy / Board of Research in Nuclear Sciences for support in carrying out the work discussed here. K Venkata Krishna is thankful to DST for fellowship and R Jyothi Lakshmi is thankful to Manipal Institute of Technology, Manipal, for granting leave for this study.

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